Genotypic assessment of Drug Resistant Tuberculosis in Baghdad and other Iraqi provinces using low-cost and density (LCD) DNA microarrays

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Running title: Genotyping of MDR TB in Baghdad and other Iraqi provinces using LCD microarrays

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Abstract

We report on a molecular investigation carried out to ascertain the prevalence of drug resistant TB and the specific gene mutations responsible for resistance to rifampicin (RIF) and/or isoniazid (INH) in Iraq. One hundred and ten clinical isolates from category II TB cases from Baghdad (58%) and several Iraqi provinces (42%) were analyzed using colorimetric, low-cost and density (LCD) microarrays (MYCO^{Direct} and MYCO^{Resist} LCD-array kits, Chipron GmbH, Germany) to identify the point mutations responsible for resistance in *Mycobacterium tuberculosis* isolates. Seventy-six patients (69.1%) had resistant strains, of which 40 (36%) were MDRTB. Where mono-resistance was identified, it was found to be predominantly to RIF (83%). The most common mutations were *rpoB* S531L (50%), *inhA* C15T (25%), and *katG* S315T (15%). The most common MDRTB genotypes were *rpoB* S531L with *inhA* C15T (60%) and *rpoB* S531L with *kat*G S315T (20%). Where phenotypic analysis of clinical isolates was also performed, genotypic data were found to show excellent correlation with phenotypic results. Correlation was found between the MYCO^{Resist} LCD-array and GenoType MTBDR*plus* for detection of resistance to RIF. Our study shows MDRTB in 36% of category II TB cases in Baghdad and surrounding Iraqi provinces which reflects World Health Organization (WHO) findings based on phenotypic studies. Diagnosis of TB and MDR-TB using culture-based tests are a significant impediment to global TB control. The LCD arrays investigated herein are easy to use, sensitive and specific molecular tools for TB resistance profiling in resource-limited laboratory settings.

Introduction

Tuberculosis (TB) is a major cause of mortality resulting in 2 million deaths globally and 9 million new cases annually (Brudey *et al.*, 2006). According to the Iraq Ministry for Health, Iraq is ranked 44th globally for TB incidence but 11th for TB mortality. Iraqi TB cases constitute 3% of total cases in the Eastern Mediterranean Region (World Health Organization (WHO), 2012b). Despite widespread application of the WHO's Directly Observed Treatment Strategy (DOTS) for TB control, TB incidence in Iraq has increased since 2003 to 117 per 100,000 population in 2010 (World Health Organization (WHO), 2012a; Yousif *et al.*, 2009). The country has been affected by conflict which has made it difficult to assess the extent of the problem. While TB incidence is high, case detection rates remain low; estimated at 43% in 2007 and 49% in 2010 (World Health Organization (WHO), 2012b). The National Tuberculosis Programme in Iraq aims to reach and maintain detection of 70% of infectious TB cases and cure at least 85% of those cases by 2013 and also to reduce the prevalence of and deaths due to TB by 50% by 2015 (Iraq Ministry for Health, 2012).

Multidrug-resistant tuberculosis (MDRTB), defined as tuberculosis showing resistance to at least isoniazid (INH) and Rifampicin (RIF), is diagnosed in >400,000 people annually (Frieden *et al.*, 1993; World Health Organization (WHO), 2007; Yew & Chau, 1995) and is a major public health problem in Iraq. Previous anti-tuberculosis therapy is the most significant predisposing factor for developing MDRTB (Sharma *et al.*, 2011). The WHO reported a worldwide MDR-TB rate of 4.3% of all TB cases in 2004 from which 29.4% were previously treated cases (Zignol *et al.*, 2006). During 2010, 3.4% of new cases and 21% of previously treated TB cases were confirmed as MDR-TB. The latest WHO global tuberculosis report 2014 described a worldwide MDR-TB rate of 3.5% in 2013 and a MDR-TB rate of 3.7% of new cases and 20% of retreatment cases in Iraq in the same year (World Health Organization (WHO), 2014); (www.who.int/tb/data; date

accessed: 15.10.2015). Ninety six percent of RIF-resistant *M. tuberculosis* occurs due to mutations in the 81bp RIF-resistance-determining region which encodes the β-subunit of RNA polymerase. The molecular mechanisms of INH resistance are more complex with over 18 genes implicated. The catalase-peroxidase gene (*katG)* is mutated in 60-70% of cases. In addition, mutations in *inhA* (which encodes a reductase used in mycolic acid biosynthesis) is associated with resistance to INH (Aragon *et al.*, 2006; Rattan *et al.*, 1998). The prevalence and type of mutations conferring resistance vary depending on geographical location (Sajduda *et al.*, 2004; Sharma *et al.*, 2003).

Since 2006 XDRTB, defined as TB resistant to one of the fluoroquinolone drugs and to at least one of the three injectable second line drugs, has been deemed by WHO to pose a serious global threat to public health, particularly in countries with a high prevalence of HIV.

Detection of mutations that confer resistance using genotypic methods is faster and lower risk than phenotypic drug susceptibility testing (DST) which requires growth of *M. tuberculosis* that can take up to several weeks (Soini & Musser, 2001). Commercial (Cinngen, Iran) and in-house end point PCR methods have been used in Iraq to detect TB, however molecular studies and evaluation of resistance patterns are rare to date (Abbas & Al-Hamdani, 2009). One study carried out in Dohuk, Iraq gave insight into the molecular epidemiology of MDRTB using spoligotyping to genotype resistant *M. tuberculosis* strains (Merza *et al.*, 2010). In this study with a limited number of 53 Iraqi patients with pulmonary TB Merza et al. identified MDR-TB in 7.9% of previously untreated patients and in 46.7% of previously treated patients. Knowledge of resistance patterns and trends is essential so that appropriate anti-TB treatment can be administered.

LCD-arrays offer an easy way to detect resistance as minimal lab instrumentation is needed. Each chip contains eight identical microarrays separated in small reaction chambers. The labelled

amplicons are hybridised to genus-specific or species-specific capture probes of 16-22 nt immobilised on the LCD-Chip surface in duplicate spots.

The MYCO^{Direct} LCD-array includes two primer pairs with 3 genus-specific capture probes, two MTBC-specific capture probes and 13 capture probes for mycobacteria other than *M. tuberculosis* (MOTT). PCR products of 226 bp result from amplification of the genus-specific internal transcribed spacer (ITS). An additional fragment of 125–165 bp from the MTBC-specific insertion element *IS6110* is also amplified depending on the species present. Due to the high degree of IS6110 sequence similarity between *M. tuberculosis* and *M. bovis*, the MYCO^{Direct} LCD-array does not allow discrimination between these species. The MYCO^{Resist} LCD-array will detect point mutations associated with drug resistance in *M. tuberculosis*. Triplex PCR results in simultaneous amplification of *rpoB*, *inhA* and *katG* genes to produce amplicons of 158 bp, 210 bp and 248 bp respectively. The capture probes for *rpoB* span a 90 bp region coding for amino acids 504–534, with 6 probes representing the wild type sequence and 12 probes representing resistance-associated SNPs. *katG* probes interrogate codon 315 as the wt sequence and two SNPs associated with INH resistance. *inhA* probes represent three point mutations associated with resistance at nucleotides 8, 15 and 17 in addition to two probes which bind to the wild type 5' non-coding region (3). The post-PCR protocol takes 45 min and results are read using a transmission-light scanner and analysed using automated software or online (www.chipron.com).

Materials and Methods

Specimen Collection

Sputum specimens were obtained from 141 Category II pulmonary TB cases over a thirteen month period (September 2008 to October 2009). All patients had failed previous TB treatment, relapsed

after treatment or defaulted during previous treatment. Definition as Category II TB was based on patient medical history including symptoms, x- ray examination, post-treatment follow-up and demographic risk factors for TB. All patients were referred to the National reference laboratories or Centre of Tuberculosis & Chest Disease in Baghdad from hospitals, health centres, and consultant clinics.

DNA from twelve MTBC isolates was received from the Scottish Mycobacteria Reference Laboratory (SMRL), Royal Infirmary of Edinburgh. Eight of these had phenotypic sensitivity results for INH and RIF. Sensitivity testing was performed using the absolute concentration method on LJ medium containing 40mg/L RIF and 0.2 mg/L INH according to WHO recommendations. Genotypic resistance to RIF and/or INH was detected by using GenoType MTBDR*plus* (HAIN Lifescience, Nehren, Germany) according to the manufacturer's instructions.

Phenotypic Characterisation

Phenotypic DST for INH and RIF were performed at the Tuberculosis and Chest Diseases Unit (Preventive Medicine and Environmental Directorate, Ministry of Health, Iraq). Direct microscopy for acid-fast bacilli (AFB) using both Ziehl–Neelsen and Auramine Phenol was performed on 110 specimens after sputum pooling and decontamination with 2% sodium hydroxide and concentration. The sensitivity of ZN stain for AFB detection is regarded to be 10^4 cfu/ml. In order to improve the sensitivity fluorescence microscopy was also applied. Three sputum specimens were used for evaluation of each patient as recommended by National guidelines of tuberculosis control (Mase *et al.*, 2007) although recent recommendations from the World Health Organization now indicate that two specimens from the patient is sufficient for diagnosis (Noeske *et al.*, 2009). Specimens were inoculated on Lowenstein–Jensen medium (LJ) prepared to the manufacturers instruction and incubated at 37°C 5-10% CO_2 for 6 weeks.

Minimum inhibitory concentration (MIC) testing was performed on 22 samples for comparison with genotypic DST results. MIC testing allowed confirmation of antibiotic resistance and establishment of the lowest antibiotic concentration that inhibited the visible growth of the microorganism after incubation. Colonies were harvested from the culture and put in a sterile tube with equal amount of sterile distilled water and mixed well, and 10^{-2} and 10^{-4} of the bacterial suspension were cultivated on LJ slants and incubated at 37ºCfor up to 2 months. Cultures were checked daily for one week, followed by once weekly for 8 weeks.

Genotypic Characterisation

DNA Extraction

DNA was extracted from each of the LJ slope culture using a freeze–thaw technique as described previously and purified using Wizard genomic DNA purification kit (Promega, Southampton, UK) (Reischl *et al.*, 1994). Genomic DNA was visualised by agarose gel electrophoresis (GelDoc 1000, Biorad, PeqPower E300, PeqLab, UK, GNA100, Amersham Bioscience). Following extraction, DNA was transported from Baghdad to UK at room temperature. DNA quantity and quality were then measured using a spectrophotometer (Nanodrop, Wilmington, DE, USA).

PCR

Multiplex PCR was performed using the labelled primers provided in the MYCO^{Direct} 1.7 and MYCO^{Resist} 3.5 LCD array kits (Chipron GmbH, Berlin, Germany) and HotStarTaq DNA Polymerase as per the manufacturer's instructions. PCR products were visualised by agarose gel electrophoresis and capillary gel electrophoresis using a Bioanalyzer 2100 (Agilent Technologies, Wokingham, UK) for enhanced sensitivity in cases where product could not be visualised on a standard gel.

Chipron LCD Arrays

MYCO^{Direct} hybridization is suited to testing of sputum samples without the need for culture providing a rapid identification of mycobacteria. In this study, culture was performed and as a result a small number of samples were tested using the species detection array. MYCO^{Resist} testing was performed on all 110 samples. Hybridization using MYCO^{Direct} and MYCO^{Resist}LCD-arrays was carried out as per the manufacturer's instructions. Briefly, biotinylated PCR product was mixed with formamide-based hybridization buffer and 30µl applied to each LCD array on the chip (Aragon, Navarro, Heiser, Garrigo, Espanol & Coll, 2006). The chip was placed on a foam pad inside a humidity chamber (containing 250 µl water). The closed chamber was transferred to a 36°C water bath for 30 min hybridisation. LCD-array chips were washed for 2 min in a low stringency buffer, placed in a Chipron transport container and dried using a centrifugation tool provided. The chips were incubated with 30µl streptavidin–horseradish peroxidase conjugate for 5 min at room temperature followed by additional wash steps (Aragon, Navarro, Heiser, Garrigo, Espanol & Coll, 2006). The container was centrifuged at 1000 rpm for 1 min to dry the chip. After drying, 30µl substrate was added to each array for a 2 min creating a dark insoluble precipitate where hybridization has taken place. High-resolution greyscale images taken using a transmission-light film scanning device were analyzed using software provided.

Hain GenoType MTBDR*plus*

GenoType MTBDR*plus* (Hain Lifescience, Nehren, Germany) simultaneously identifies MTBC and resistance to RIF and/or INH from cultured isolates or smear-positive pulmonary specimens. PCR products are hybridised to a nitrocellulose strip containing 27 reaction zones including 11 *rpoB, katG* and *inhA* wild type probes and 10 mutation probes to detect some of the most common mutations that confer resistance. The mutation probes for *rpoB* are designed to detect D516V (*rpoB* MUT1), H526Y (*rpoB* MUT2A), H526D (*rpoB* MUT2B) and S531L (*rpoB* MUT3). Two capture probes for the *katG* gene harbour the sequence for the mutations S315T1

(*katG* MUT1) and S315T2 (*katG* MUT2). The capture probes for *inhA* promoter region have been designed for four point mutations C15T, A16G, T8C, T8A. Following the manufacturer's instructions, GenoType MTBDR*plus* was performed on eight DNA samples at SMRL and the results made available for this study.

Results

Iraq TB study panel

The geographical distribution of 110 patients included in the study is depicted in Figure 1A. Eighty-two patients (75%) were from Baghdad or the bordering provinces. Patient's age ranged from 11-70 years but more than half of the TB cases (56%) were in the 20-40 age category (Figure 1B). TB was slightly more prevalent among males than females included in the study (68 % and 32 % respectively (Figure 1C). All 141 sputum samples were smear-positive using one or both AFB staining methods. Thirty-one specimens were excluded from analysis due to contamination of the LJ media or because the mycobacteria failed to grow.

MYCO^{Direct} and MYCO^{Resistance} LCD-array

Transport of gDNA resulted in loss of DNA integrity in some samples (data not shown). However, the concentration of extracted DNA ranged from 22.5ng/µl to 657 ng/µl with A260/280 purity of 1.6 to 2 which was considered suitable for the study. *IS6110* PCR was positive for all 110 samples amplified with MYCO^{Direct} primers indicating *M. tuberculosis*. Thirty-nine samples which were hybridized on MYCO^{Direct} microarrays yielded a positive result for *M. tuberculosis*. Some variability was seen in intensity of the colourimetric microarray signals due to differences in the concentration of template DNA used in the PCR.

Hybridisation of triplex *rpoB, inhA* and *katG* PCR products to the MYCO^{Resist} LCD-array showed that 34 (31%) MTBC isolates were sensitive to both RIF and INH (Figure 3). Seventy five (69%) MTBC isolates contained SNP mutations conferring a range of resistance genotypes (Table 2 and Figure 4). Monoresistant strains made up 32% of isolates tested, of which 83% were RIF resistant. The most common mutations found in each resistance gene were *rpoB* S531L (50% frequency), *inhA* C15T (25%), and *katG* S315T (15%). Other SNPs were found at *katG* S315N (5%), *rpoB*

L533P (5%), *rpoB* H526Y (4%), *rpoB* D516V (3%), *rpoB* S531W (2%) and *rpoB* H526D (2%). Of the resistant isolates, 40 (36%) were found to be multidrug-resistant. The most common MDRTB genotypes were *rpoB* S531L with *inhA* C15T (60% of the multidrug-resistant isolates) and *rpoB* S531L with *katG* S315T (20%). A greyscale image showing typical hybridisation signals for MDRTB (clinical isolate 16) using the MYCO^{Resist} LCD-array is shown in Figure 2A. Figure 2B shows the corresponding colour intensity of the hybridisation signal for each feature with SNPs identified at *rpoB* S513L and *inhA* C15T.

Comparison of MYCO^{Resist} LCD-array and GenoType MTBDR*plus* for the detection of resistance to RIF and/or INH

RIF resistance or susceptibility was correctly identified in all DNA extracts provided by SMRL based on results of MYCO^{Resist} LCD-array and the GenoType MTBDR*plus* analysis. In five of 8 (63%) SMRL samples, mutations identified were concurrent between MYCO^{Resist} LCD-array and GenoType MTBDR*plus* (Table 1). A mutation conferring resistance to INH was not detected by the LCD-array in two MDR-TB samples and GenoType MTBDR*plus* also failed to detect a INH resistance mutation. Another discrepancy was found in one sample due to the identification of an incorrect SNP in *inhA*. To assess the sensitivity and specificity of both methods a larger sample number would be required but this was not an aim of this study.

Genotypic compared with phenotypic DST

Where comparison is possible (20%/22 isolates), MYCO^{Resist} results showed also good correlation with phenotypic DST results (Table 2). As a result of time demands of DST and limited resources, testing analysis of all samples was not undertaken and twenty percent of study samples were considered adequate for robust comparison with genotypic data. Using the molecular test, a *rpoB* mutation was found in 15 clinical isolates which had also been determined to be RIF-resistant by

phenotypic methods. Similarly, 10 clinical isolates which were found to be INH-resistant using phenotypic methods gave a positive hybridisation signal for an *inhA* or *katG* mutation using the MYCO^{Resist} array. No mutations were found by genotypic analysis in 6 MTBC which were fully sensitive to both INH and RIF by phenotypic DST. Only one sample (clinical isolate 34) gave a discrepant result. Relative to phenotypic DST analysis, isolate 34 yielded a false positive result of INH resistance.

Discussion

Diagnosis of TB and MDRTB, particularly in developing countries is based on time consuming, culture-based tests that have been in use for many decades and are a significant impediment to global TB control. Rapid detection of TB drug resistance is critical to patient care but the turnaround of culture-based DSTs diminishes their clinical impact. LCD arrays allow more rapid turnaround times and potentially faster initiation of appropriate treatment which may reduce spread of TB disease. In Iraq, the emergence of resistance and in particular MDRTB has become a major public health problem (World Health Organization (WHO), 2012b). Application of the easy to use molecular method, suitable for use in resource-limited laboratory settings, has facilitated clinical evaluation of MDRTB of samples from Baghdad (58%) and a broad geographical distribution of Iraqi provinces (42%). The study has yielded data on prevalence which reflects benchmark methods and WHO data, and additional data on frequency of resistance genotypes.

Our SNP frequency findings are in keeping to those reported around the world. The *rpoB* S531L SNP is the most common mutation site associated with RIF resistance, but the frequency of other *rpoB* mutations varies depending on geographical location (Kapur *et al.*, 1994; Mani *et al.*, 2003; Pozzi *et al.*, 1999; Soudani *et al.*, 2007). *rpoB* mutations at position H526Y and S512T feature at high frequency in many regions, and this is not seen herein (Cavusoglu *et al.*, 2002; Sajduda,

Brzostek, Poplawska, Augustynowicz-Kopec, Zwolska, Niemann, Dziadek & Hillemann, 2004). Although *katG* codon315 was the only site on this gene interrogated in this study, mutations at this position have been frequently reported in both INH-resistant, and MDRTB strains (Hillemann *et al.*, 2005; Mokrousov *et al.*, 2002; van Soolingen *et al.*, 2000; Viader-Salvado *et al.*, 2003). A number of isolates in this study were found to have a SNP at *inhA*C15T. This mutation has also been reported in various geographical locations, but is considered responsible to a lesser extent for INH resistance (Bakonyte *et al.*, 2003; Jiao *et al.*, 2007; Wu *et al.*, 2006). Frequency distribution of SNPs identified in our study are corroborated in many European genotypic MDRTB studies (Hillemann, Weizenegger, Kubica, Richter & Niemann, 2005; Sajduda, Brzostek, Poplawska, Augustynowicz-Kopec, Zwolska, Niemann, Dziadek & Hillemann, 2004). MDRTB resistance profiles and prevalence identified herein are similar to other findings in the WHO Eastern Mediterranean Region. For example, in Saudi Arabia, SNP frequencies and incidence of MDRTB levels are also similar at 39%, although a different population tested (Nimri *et al.*, 2011). A recent genotypic study in Jordan found *rpoB* S531, *rpoB*516, *rpoB*526and *katG* to be of highest frequency, with incidence of MDRTB at 6% based on newly diagnosed TB cases. Similar SNP frequency distribution findings were reported in Iran (Khosravi *et al.*, 2012). According to the WHO, MDRTB is found in 8.1% (Turkey), 9% (Saudi Arabia), 26% (Syria), 29% (Iran), and 48% (Jordan) of Category II TB cases (World Health Organization (WHO), 2012c). Our study shows MDRTB in category II TB cases in Iraq to be amongst the highest prevalence countries in the region.

In conclusion, the MYCO^{Resist} LCD-array was successfully employed to identify the frequency of specific *rpoB*, *katG* and *inhA* mutations that confer resistance in Iraqi category II TB cases. The test is simple, cost effective and rapid. Results showed widespread occurrence of mono-resistant genotypes and MDRTB. A genotypic study of this nature provides insight into drug resistance patterns for this region in which prevalence of drug resistant TB is an ongoing problem.

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Figure captions

Figure 1: Geographical distribution of samples within provinces of Iraq (a) (map of Iraqi provinces downloaded from http://www.d-maps.com/carte.php?num_car=4295&lang=en; 02 November 2015). (b) shows age distribution of patients included in the study. (c) shows gender distribution within the study (35 female patients and 75 male patients).

Figure 2: An example of high-resolution grey-scale image (top right) taken using a transmission-light film scanning device (Chipron GmbH, Germany) of the MYCO^{Resist} array following hybridization of clinical isolate 16. The top right figure shows the quantified intensity values.

Figure 3: Frequency distribution of genotypic antibiotic resistance detected in 110 clinical isolates from Iraqi category II TB patients studied.

Figure 4: Frequency distribution of SNPs identified amongst three genes investigated in 110 clinical isolates tested. Highest frequency is identified in *rpoB* S513L, *inhA*C15T and *katG* S315T.

450 **Table 1:** Comparison of MYCO^{Resist} LCD-array and GenoType MTBDR*plus* for detection of

451 resistance to RIF and/or INH:

SMRL Lab No. Group B	Phenotypic DST	Resistance mutation detected by GenoType MTBDR*plus*	Resistance mutation detected by MYCO^{Resist} LCD-array
MR216581T (A)	RIF-S, INH-R	*katG* MUT1 (S315T1) AGC→ACC	*katG*S315N AGC→AAC
MR171425H (B)	RIF-S, INH-R	*katG*MUT1 (S315T1) AGC→ACC *inhA* MUT1 (C15T)	*katG* S315N AGC→AAC *inhA* C15T
MR122781F (C)	RIF-R, INH-S	*rpoB* MUT3 (S531L) TCG→TTG	*rpoB* S531L TCG→TTG
MR151475B (D)	RIF-R, INH-R	*rpoB* MUT3 (S531L) TCG→TTG *katG*MUT1 (S315T1) AGC→ACC	*rpoB* S531L TCG→TTG No *inhA* mutation detected
MR199029V (E)	RIF-S, RIF-S	None	no mutation
MR135612M (F)	RIF-R, INH-R	*rpoB*MUT3 (S531L) TCG→TTG	*rpoB* S531L TCG→TTG
MR164347P (G)	RIF-S, INH-R	*inhA* MUT3B (T8A)	*inhA* C15T
MR188497P (H)	RIF-S, INH-R	*inhA* MUT1 (C15T)	*inhA* C15T

452

453 **Table 2:** Genotypic and phenotypic drug susceptibility results for 110 tested isolates:

Sample ID	Mutation detected by MYCO^{Resist}	Phenotypic Resistance INH\RIF	Sample ID	Mutation detected by MYCO^{Resist}	Phenotypic Resistance INH\RIF
1	*katG*S315T	NA	56	*rpoB* S531L	NA
2	*katG*S315T, *rpoB* S531L	NA	57	*rpoB* S531L	NA
3	No mutation detected	NA	58	No mutation detected	NA
4	*inhA* C15T, *rpoB* S531L	NA	59	*rpoB* D516V	NA
5	No mutation detected	NA	60	No mutation detected	NA
6	*rpoB* S531L	S\R	61	*rpoB* H526Y	NA
7	*rpoB* H526Y	S\R	62	No mutation detected	NA
8	*inhA* C15T, *rpoB* S531L	NA	63	*rpoB* S531L, *inhA* C15T	NA
9	No mutation detected	NA	64	*rpoB* S531L, *inhA* C15T	NA
10	*rpoB* S531L	NA	65	No mutation detected	NA
11	*katG*S315T, *rpoB* D516V	R\R	66	*rpoB* S531L, inhA C15T	NA
12	*rpoB* H526Y, *katG*S315N	R\R	67	*rpoB* S531L	NA
13	No mutation detected	S\S	68	*rpoB* S531L	NA
14	No mutation detected	NA	69	*rpoB* S531L, *katG*S315N	NA

15	*rpoB* S531L	NA	70	*rpoB* S531L	NA
16	*rpoB* S531L, *inhA* C15T	NA	71	*rpoB* S531L	NA
17	*rpoB* S531L, *inhA* C15T	NA	72	*rpoB* L533P, *katG*S315T	NA
18	*rpoB* S531L, *inhA* C15T	R\R	73	*rpoB* S531L, *katG*S315T	NA
19	*rpoB* S531L, *inhA* C15T	R\R	74	*rpoB* S531L, *inhA* C15T	NA
20	No mutation detected	S\S	75	No mutation detected	NA
21	*rpoB* S531L	S\R	76	*rpoB* S531W, *rpoB* S531L	NA
22	*rpoB* S531L	NA	77	*rpoB* H526D, *katG*S315N	NA
23	*rpoB* S531L	NA	78	*katG*S315T, *rpoB* S531L	NA
24	*rpoB* L533P, *inhA* C15T	NA	79	*katG*S315T	NA
25	*rpoB* S531L, *inhA* C15T	NA	80	*rpoB* S531L, *inhA* C15T	NA
26	No mutation detected	NA	81	No mutation detected	NA
27	No mutation detected	S\S	82	*rpoB* S531L	NA
28	No mutation detected	NA	83	*katG*S315T	NA
29	No mutation detected	S\S	84	No mutation detected	NA

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30	*katG*S315T	R\S	85	No mutation detected	NA
31	*rpoB* S531L, *inhA* C15T	R\R	86	*rpoB* S531L, *inhA* C15T	NA
32	*rpoB* S531L, *inhA* C15T	S\R	87	*rpoB* S531L, *katG*S315T	NA
33	*rpoB* S531L	R\R	88	No mutation detected	NA
34	*rpoB*L531L, *inhA* C15T	S\R	89	No mutation detected	NA
35	*rpoB* L533P, *katG*S315T	NA	90	*rpoB* S531L, *katG*S315T	NA
36	*rpoB* S531W	NA	91	*rpoB* S531L	NA
37	*rpoB* S531L, *inhA* C15T	NA	92	No mutation detected	NA
38	*rpoB* L533P, *inhA* C15T	NA	93	No mutation detected	NA
39	*rpoB* S531L, *inhA* C15T	NA	94	*rpoB* S531L	NA
40	No mutation detected	NA	95	*rpoB* S531L	NA
41	No mutation detected	NA	96	No mutation detected	NA
42	*rpoB* S531L, *inhA* C15T	R\R	97	No mutation detected	NA
43	*rpoB* H526D, *katG* S315T	R\R	98	*rpoB* S531L, *inhA* C15T	NA
44	*rpoB* S531L, *inhA* C15T	NA	99	*rpoB* S531L, *inhA* C15T	NA

455 NA: Not applicable, testing not performed, R= Resistant, S= Sensitive.

45	No mutation detected	S\S	100	No mutation detected	NA
46	No mutation detected	NA	101	No mutation detected	NA
47	*rpoB* S531L	NA	102	No mutation detected	NA
48	*rpoB* S531L, *katG*S315T	R\R	103	*rpoB* S531L	NA
49	No mutation detected	S\S	104	*rpoB* S531L	NA
50	*rpoB* D516V	S\R	105	*rpoB* S531L	NA
51	*rpoB* S531L, *inhA* C15T	NA	106	*rpoB* H526Y,*katG* S315N	NA
52	*rpoB* S531L, *inhA* C15T	NA	107	*rpoB* S531L, *katG*S315T	NA
53	No mutation detected	NA	108	*rpoB* S531L, *katG*S315T	NA
54	No mutation detected	NA	109	*katG*S315T, *inhA* C15T	NA
55	No mutation detected	NA	110	*katG*S315T	NA

Figure 1 (a)

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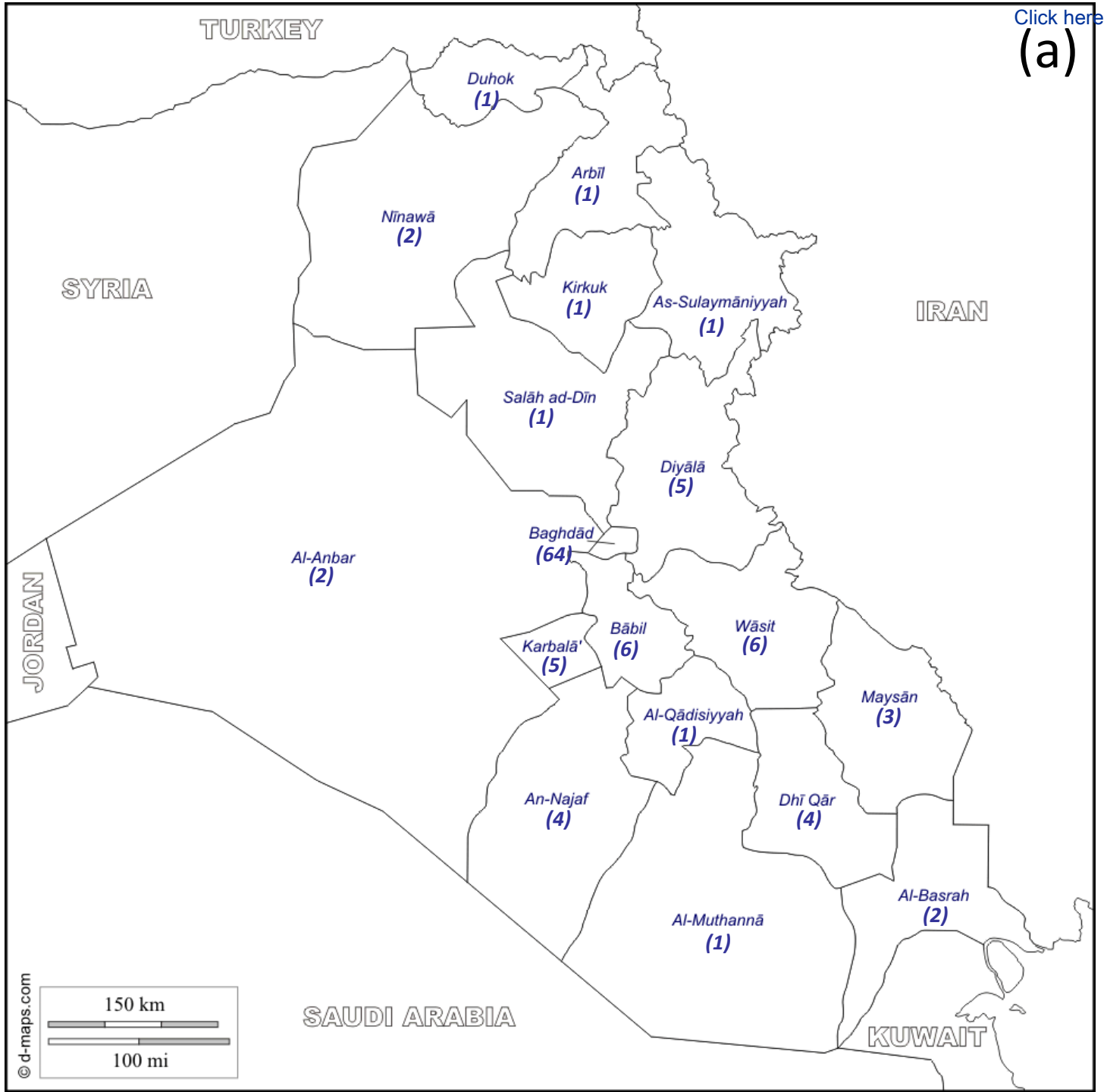

Figure 1 (b)

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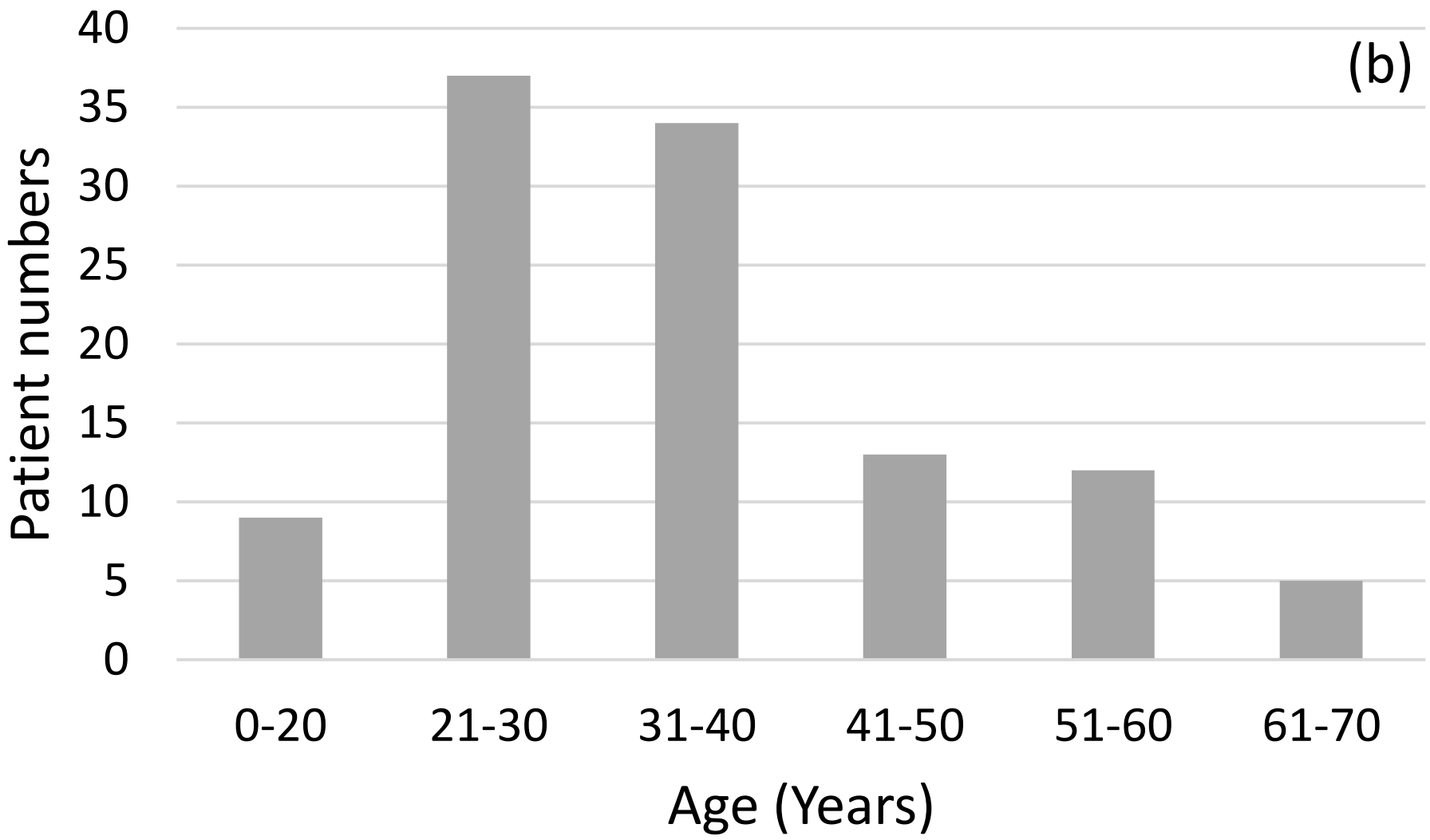

Figure 1 (c)

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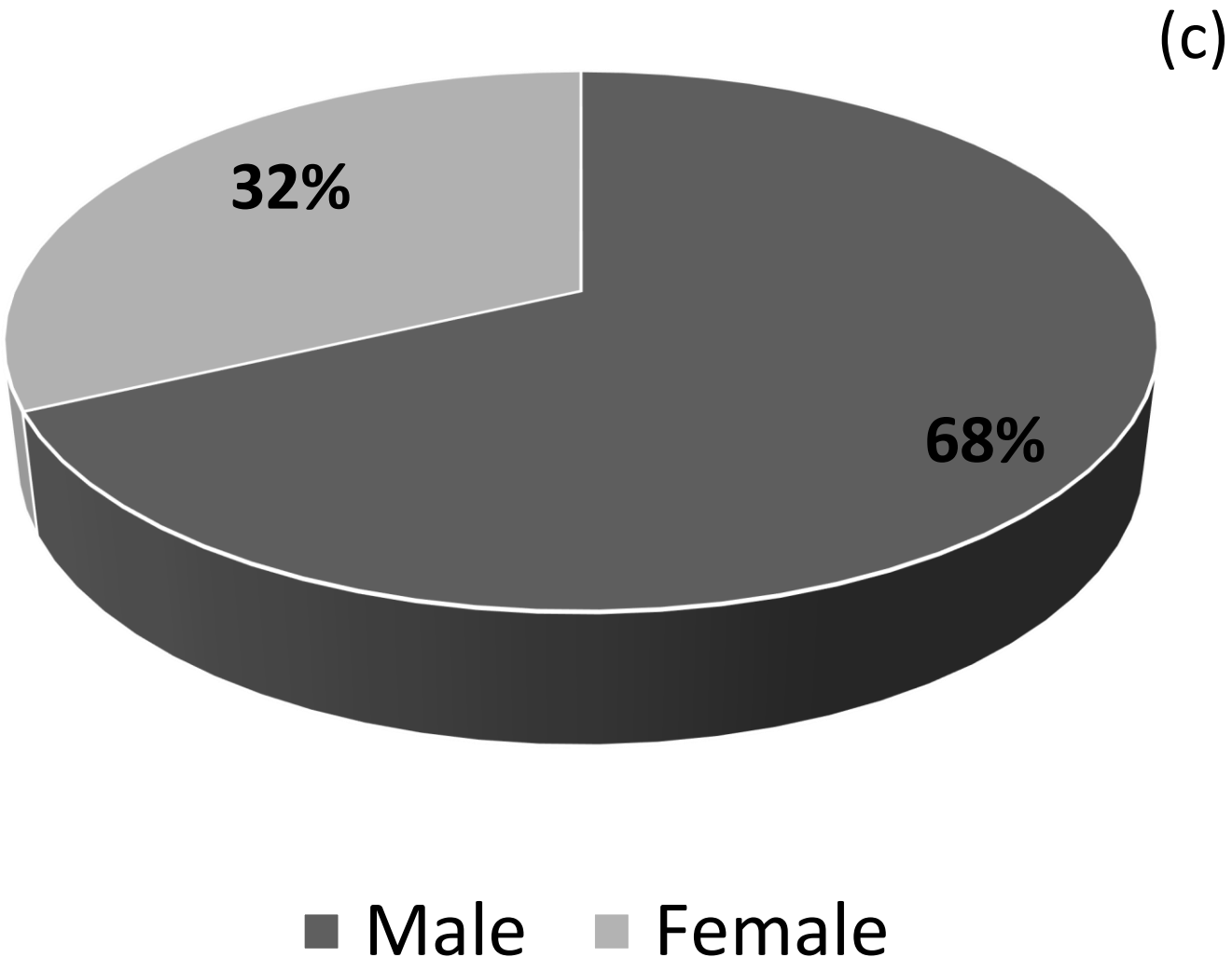
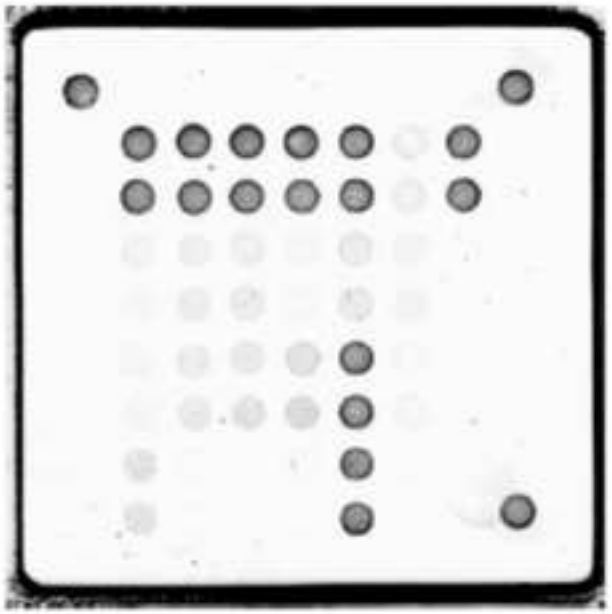
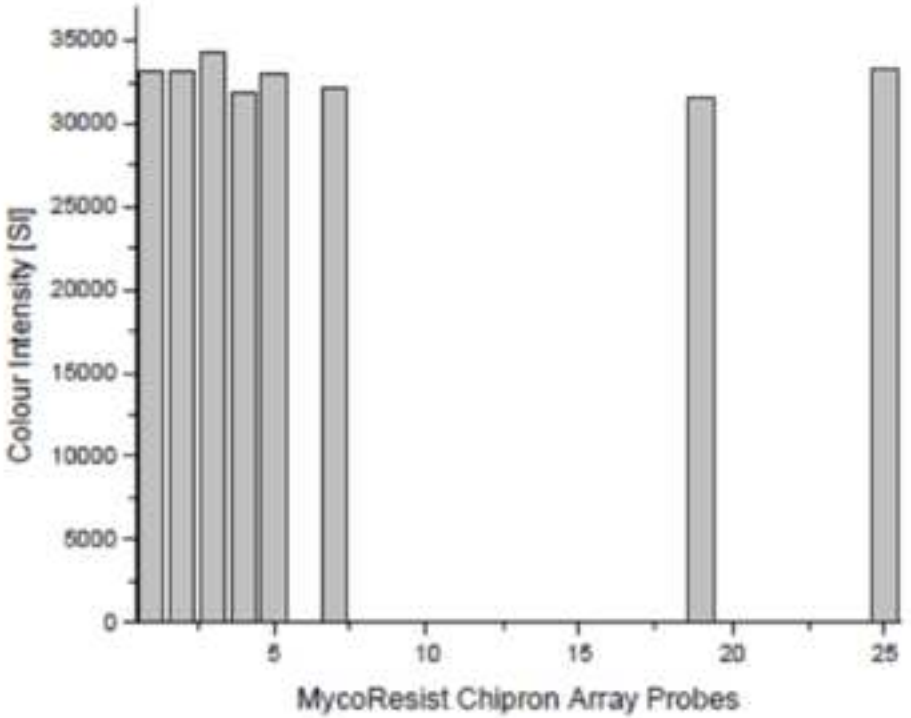
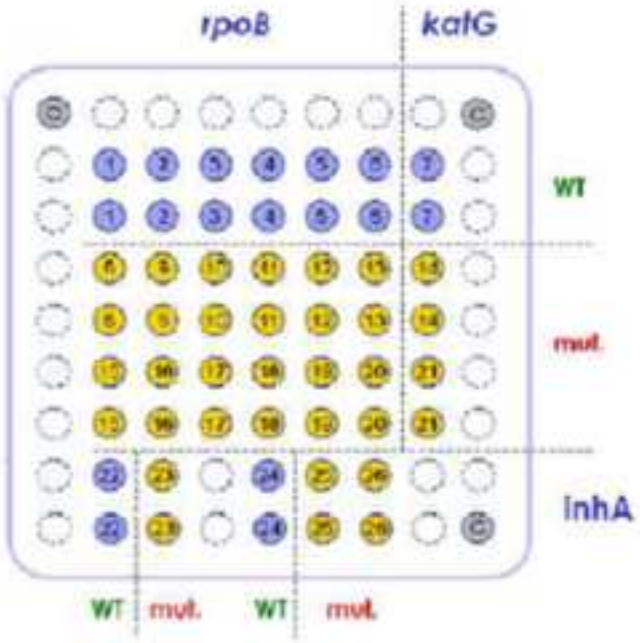


Figure 2







No	Probe Specificity	Gene
1	WT-AA-504-509	*rpoB*
2	WT-AA-509-515	*rpoB*
3	WT-AA-514-519	*rpoB*
4	WT-AA-526-524	*rpoB*
5	WT-AA-525-530	*rpoB*
6	WT-AA-530-534	*rpoB*
7	WT-315-Ser	*katG*
8	Mut-511-Pro	*rpoB*
9	Mut-516-Tyr	*rpoB*
10	Mut-526-Asn	*rpoB*
11	Mut-526-Asp	*rpoB*
12	Mut-526-Arg	*rpoB*
13	Mut-531-Trp	*rpoB*
14	Mut-315-Thr	*katG*

No	Probe Specificity	Gene
15	Mut-512-Thr	*rpoB*
16	Mut-516-Val	*rpoB*
17	Mut-526-Leu	*rpoB*
18	Mut-526-Tyr	*rpoB*
19	Mut-531-Leu	*rpoB*
20	Mut-533-Pro	*rpoB*
21	Mut-315-Asn	*katG*
22	WT nt -8	*inhA*
23	Mut -8-C	*inhA*
24	WT nt -15-17	*inhA*
25	Mut -15-T	*inhA*
26	Mut -17-T	*inhA*
C	Hyb-Control	-

Figure 3

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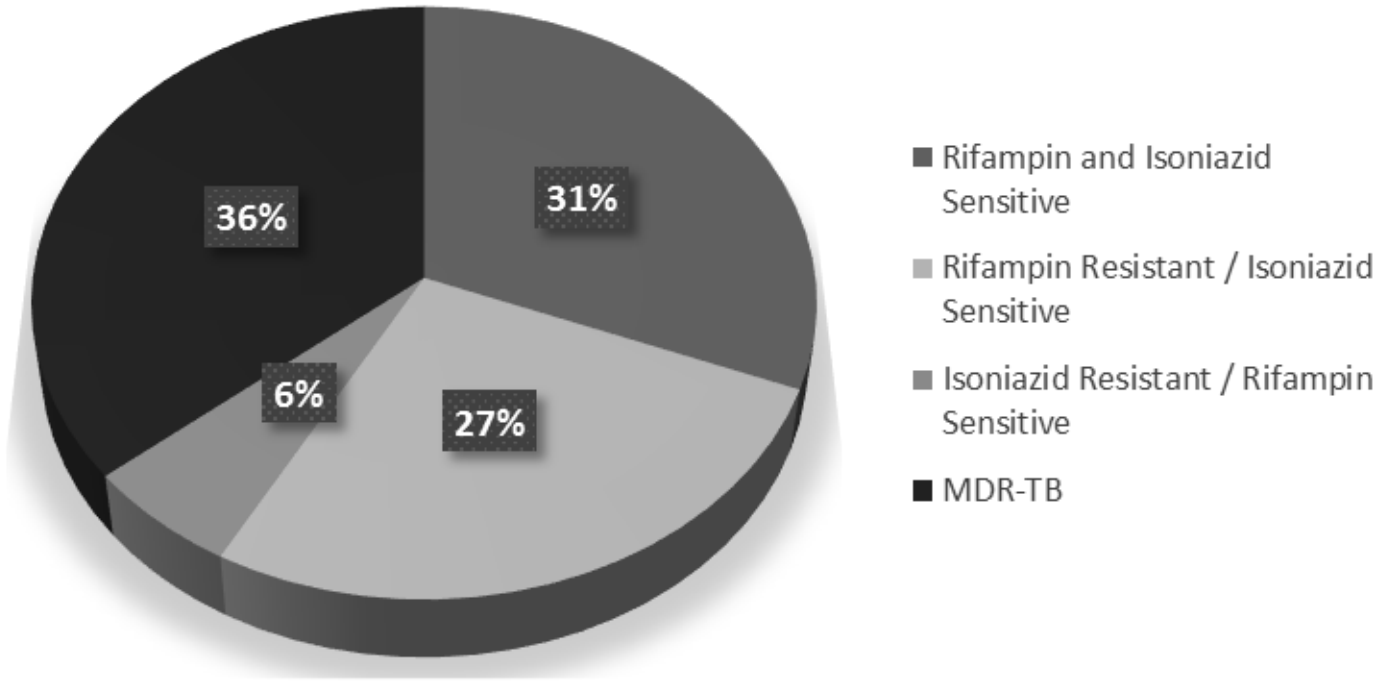

Figure 4

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